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14. ABSTRACT

With the emergence of CMLs that are resistant to Bcr-Abl tyrosine kinase inhibitors, it becomes imperative that we identify other effective agents to eliminate these cells. In recent years, it has become evident that most successful chemotherapeutics work by inducing apoptosis. Unfortunately, high levels of Bcr-Abl can preclude the successful use of many agents, by dampening the apoptotic response. In this proposal, we developed a strategy that relies on, indeed exploits, the high tyrosine kinase activity of Bcr-Abl to induce cell killing. We have designed and engineered constructs to fuse the catalytic domains of caspases (the apoptotic proteases) to either an SH2 domain or to sites well-phosphorylated by Bcr-Abl. We have made variants based on the Crk SH2 domain as well as phosphorylation sites from Stat 5, Bcr-Abl, and Crk itself (this is the sequence to which the Crk SH2 domain would bind intramolecularly). These have now been used to infect cells. Initial results suggest that the relevant fusion proteins are being produced and that there may be selective killing of Bcr-Abl-expressing cells.

15. SUBJECT TERMS
CML, caspases, Bcr/Abl, SH2, phosphotyrosine

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Introduction

Apoptosis is a cellular suicide program initiated by a host of stimuli, including DNA damage, binding of particular extracellular ligands or growth factor withdrawal.
Bcr-Abl, the activated kinase created by chromosomal translocation in chronic myelogenous leukemia (CML), can both induce cellular proliferation and confer resistance to apoptotic cell death. As most chemotherapeutic agents act, at least in part, by inducing apoptosis, this resistance to apoptosis presents a problem for the chemotherapeutic elimination of CML cells. **The objective of our funded grant was to develop a novel and innovative method to selectively kill CML cells through the forcible induction of apoptosis. Our approach was aimed at eliminating Bcr-Abl-expressing cells, while sparing their normal counterparts.**

Our overall strategy was to design and validate variants of the apoptotic proteases, caspase 8 and 9, whose dimerization (and hence activation) was **dependent** upon a high level of Bcr-Abl tyrosine kinase activity. As Bcr-Abl normally prevents apoptosis by blocking the activation of caspases 8 and 9, such variants would bypass the protective mechanism and kill Bcr-Abl expressing cells. Because Bcr-Abl is a kinase unique to CML cells, these variants should remain harmless in normal cells.

Body of Report

The principle behind our proposal was to design caspase variants that could only be activated in Bcr-Abl-transformed cells. This was to be done by taking the catalytic domain of the caspase (8 or 9) and replacing the pro-domains with either an SH2 domain (or other phosphotyrosine-binding peptide) or a site that would be well phosphorylated in Bcr-Abl-expressing cells. Dual infection of cells with both constructs would result in an interaction between the SH2 domain on one construct and the phosphorylated tyrosine on the other construct, promoting selective dimerization of the caspases in the Bcr-Abl-infected, but not control, cells. Towards this end, we proposed in Task I to design and engineer the variants, in Task 2 to perform tissue culture assays in and Task 3 to assay the constructs in mice. We have completed Task I, as summarized below, have made progress in Task 2 and are continuing our work to undertake Task 3, once Task 2 is completed.

Task 1:
We proposed to select SH2-phosphopeptide pairs and/or select novel peptides able to bind tyrosine phoshporylated Bcr-Abl substrates. We prepared GST-SH2 domains from PLC-g, RAS-GAP, LCK, Crk, PI3K, NCK, GRB2, and Bcr-Abl to compare their relative efficiencies at binding tyrosine phosphorylated proteins from Bcr-Abl, but not control cell lysates. A number of these bound a range of tyrosine phosphorylated proteins in Bcr-Abl-transformed but not control cell lysates. Because we had a range of Crk constructs in the lab and because Crk most consistently bound the tyrosine phosphorylated proteins, we selected the Crk SH2 domain to go forward. At the same time, we prepared DNAs encoding the Tyr phosphorylation sites of Crk (Tyr 221) and Stat 5 (Tyr 694), the autophosphorylation stie of Bcr-Abl, and, eventually, a model Bcr-Abl substrate based on the optimum consensus phosphorylation site (AVIYAAPF).

We next proposed to construct plasmid-encoded fusions between the Crk SH2 and caspase 8 or SH2-caspase 9 as well as the tyrosine phosphorylation site variants bound to caspase 9. These

were constructed and transfected into several cultured cell lines. Although the proteins were expressed, the transfection efficiency was so poor, we could not tell if the constructs were killing cells in any specific way. Hence, we moved directly onto construction of viral vectors. We first produced MSCV-IRES GFP and DsRED constructs, but found that the DsRES was not sufficiently bright for reproducible sorting by the Duke FACS facility. Hence, we have now switched to YFP and CFP tags. We have constructed all of the variants described above.

Task 2

As described above, plasmid constructs did not prove promising. Thus, we have retrovirally infected Bcr-Abl and normal cells with the constructs described above and have some initial promising indication that our strategy is working to selectively kill the Bcr-Abl-transformed cells in culture. We are preparing to repeat these experiments with more rigorous quantitation, using multiple parameters for measurement of apoptosis. If these experiments are successful ,we will proceed to task 3 with whole animal models.

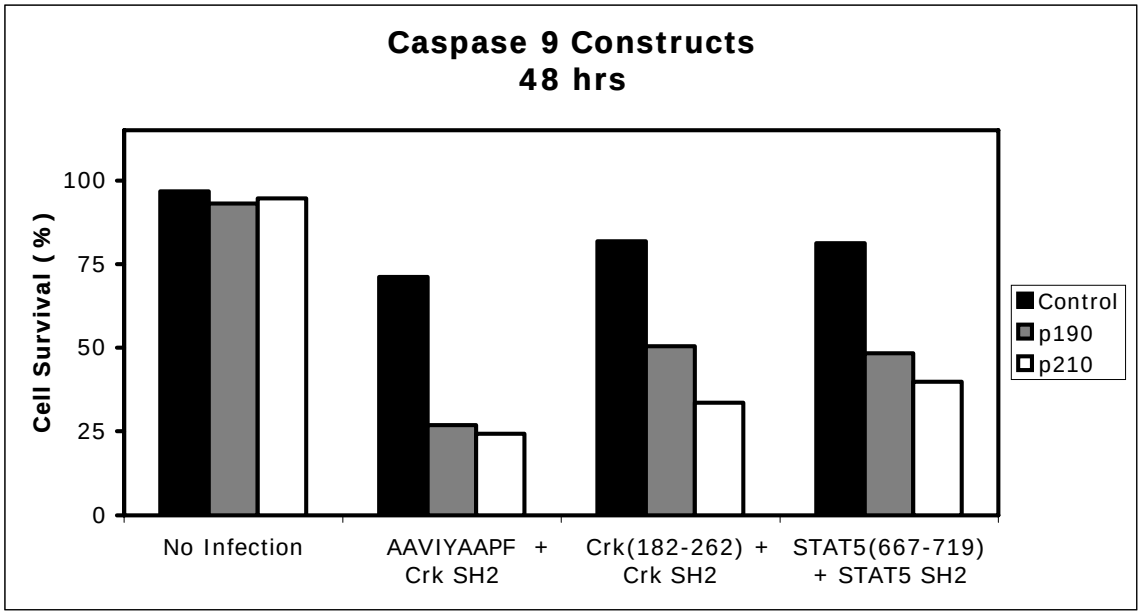

Fig. 1. Bcr-Abl-expressing or control cells were dual-infected with the caspase 9 variants shown above. Cell death was quantitated by PI staining and flow cytometry.

Key Research outcomes

- Design and engineering of plasmid vectors for the selective killing of Bcr-Abl cells.
- Initial expression testing of proteins.
- Transfer of Crk-based constructs into MSCV vector and initial expression testing
- Initial killing assays

Reportable outcomes

None as yet, but award was only held for 18 months. Further reportable results are anticipated in the next 12 months.

Conclusions

Despite numerous technical difficulties, we have finally engineered a set of promising viral vectors with the potential to yield selective killing of Bcr-Abl-expressing cells. This represents a novel strategy to kill these cells. Moreover, if we can confirm that our new constructs are successful in inducing selective killing of the Bcr-Abl-expressing cells, this will be the first example of conditional caspase activation depending on the transformed state of the cell.